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TECHNICAL ADVANCE

# A chimeric transactivator allows tetracycline-responsive gene expression in whole plants

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## Summary

**The chimeric transcriptional activator tTA, a fusion between the Tn*10* encoded Tet repressor and the activation domain of the *Herpes simplex* virion protein VP16, was stably expressed in transgenic tobacco plants. It stimulates transcription of the β-glucuronidase (*gus*) gene from an artificial promoter consisting of 7 *tet* operators and a TATA-box. Tetracycline, which interferes with binding of tTA to operator DNA, reduces *gus* expression over several orders of magnitude. This stringency of regulation suggests that the system can be used to construct transgenic plants encoding a potentially lethal gene product. Furthermore, the specific and fast inactivation of tTA allows study of the stability of RNAs and proteins.**

## Introduction

Expression of foreign genes in transgenic plants is a widely used tool to confer new characters to different species (Schell, 1987; Willmitzer, 1988). In addition, enhancing or reducing the expression of endogenous genes helps us to understand the contribution of a defined gene product to the phenotype. The ability to control expression of a gene via a highly specific mechanism offers unique opportunities to study the physiological functions of certain gene products at different stages of development. The correlation of the phenotype with the kinetics of induction allows differentiation between primary and secondary consequences, which generates another advantage of a regulated expression system. Moreover, a stringently regulated promoter is absolutely required, if the expression of a gene product of interest interferes with the regeneration process.

Ideally, an inducible promoter should show extremely low or no basal levels of expression in the absence of inducing conditions, a high level of expression in the induced stage, and an induction scheme that does not otherwise alter the physiology of the plant. The last requirement, especially, renders the use of endogenous promoters, that respond to stimuli like heat (Schöffl *et al.*, 1989), wounding (Keil *et al.*, 1989), nitrate (Back *et al.*, 1991) or light (Gilmartin *et al.*, 1990) less favorable. A more promising approach is to combine regulatory control elements from other organisms, that respond to signals usually not encountered by a plant, with the general plant transcription machinery.

Based on this idea, two different concepts of gene control can be realized, i.e. promoter-repressing systems and promoter-activating systems. One way to construct a promoter repressing system is to use bacterial repressors to compete directly with plant transcription factors and/or RNA polymerases for binding (Gatz *et al.*, 1992; Wilde *et al.*, 1992). Using the Tn*10* encoded Tet repressor (TetR) in combination with a suitably engineered Cauliflower Mosaic Virus (CaMV) 35S promoter with three integrated *tet* operator sites we have succeeded in constructing a tightly repressible expression system (Gatz *et al.*, 1992). The DNA-binding affinity of TetR can be abolished by low amounts of tetracycline (Tc). The high equilibrium association constant of about $10^{-9}$M for the repressor–inducer complex (Takahashi *et al.*, 1986) ensures efficient induction at Tc concentrations that do not even inhibit the growth of procaryotes (Geissendörfer and Hillen, 1990). Addition of Tc leads to a 200- to 500-fold induction of promoter activity throughout intact tobacco plants without any obvious inhibition of plant growth (Roeder *et al.*, 1994).

In this paper we describe the characterization of a Tc-dependent expression system in transgenic plants that combines the features of TetR with those of a promoter-activating system. Because of the modular organization of transcription factors (Frankel and Kim, 1991), eucaryotic activation domains can be fused to procaryotic repressor proteins thus turning them into transcriptional activators (Brent and Ptashne, 1985; Labow *et al.*, 1990). Fusion of

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TetR to the activation domain of virion protein 16 (VP16) of *herpes simplex* virus did not significantly alter the DNA-binding properties and the Tc inducibility of the TetR moiety (Gossen and Bujard, 1992). In Hela cells, this chimeric transcriptional activator (tTa) stimulated transcription from a minimal promoter sequence combined with *tet* operator sequences. Addition of Tc reduced gene expression over several orders of magnitude down to background levels.

## Results

### *The Tc-controlled transcriptional activator tTA is functional in transiently transformed plant cells*

To establish a gene expression system that can be negatively regulated by Tc we started with the construction of two types of DNA constructs termed 'activator plasmids' and 'target plasmids'. The 'activator plasmid' contains the chimeric *tTA* gene encoding amino acids 1–207 of TetR (Postle *et al.*, 1984) fused in frame to amino acids 363–490 of the transcriptional activator VP16 (Triezenberg *et al.*, 1988) under the control of the CaMV 35S promoter (Benfey *et al.*, 1990; Figures 1 and 2). The 'target plasmid' was made by replacing the enhancer sequences of the CaMV 35S promoter (sequences upstream from position –53) by a DNA fragment containing seven *tet* operator sites (Figures 1 and 2). The capacity of tTA to activate gene expression in plant cells was assessed by measuring Gus activity in extracts prepared from tobacco cells transfected with the 'target plasmid pUC-Top10' and increasing amounts of the 'activator plasmid pUC-TetVP16'. The left panel of Figure 1 contains five pieces of information;

(i) the VP16 activation domain is able to stimulate transcription in tobacco cells;

(ii) increasing the amount of 'activator plasmid' beyond an optimal level leads to a decrease in transcriptional activation;

(iii) when given optimal levels of 'activator plasmid', expression levels are twofold to threefold higher than those mediated by the CaMV 35S promoter;

(iv) in the absence of the 'activator plasmid', the 'target construct' mediates levels of gene expression which range between 10 and 18% of the maximal level obtained with optimal amounts of the 'activator plasmid';

(v) Tc reduces activation by tTA. When added at concentrations larger than 7 mg $l^{-1}$ the residual Gus activity amounts to roughly the same level as mediated by the 'target plasmid' alone.

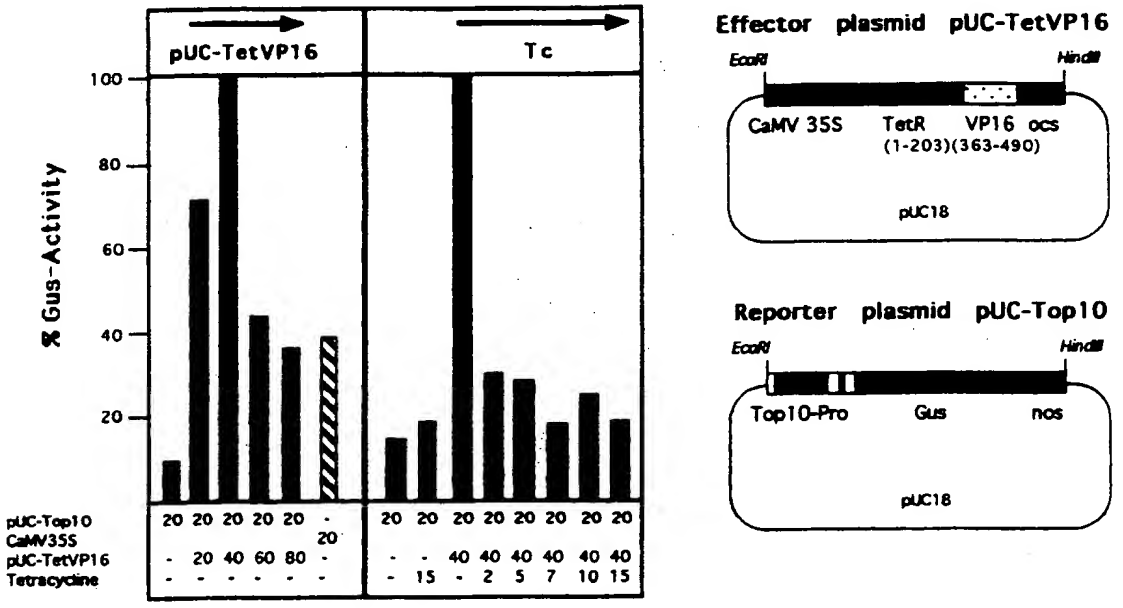


**Figure 1.** Transient analysis of tTa activity in tobacco protoplasts.

Left panel: relative Gus levels showing tTA function in plant protoplasts. The Gus levels detected with the various plasmid constructs are all shown as a percentage of the amount obtained when cells were co-transfected with 20 µg pUC-Top10 and 40 µg pUC-TetVP16. Below the graph the amount of the transfected plasmids is indicated in µg. pAT1 contains a CaMV 35S promoter derivative 5' to the *gus* gene (Gatz *et al.*, 1991, pAT1 is pAT2, except that pUC18 is the vector). Sheared salmon sperm DNA (carrier) was added such that plasmid and carrier DNA totalled 120 µg. The amount of Tc is given in mg $l^{-1}$. In the experiment showing the effect of Tc, six batches of protoplasts transfected with 20 µg pUC-Top10 and 40 µg pUC-TetVP16 were combined after PEG treatment and divided afterwards to allow cultivation with increasing amounts of Tc. Black columns; expression levels of the Top10 promoter; shaded column; expression level of the CaMV 35S promoter.

Right panel: maps of the 'activator plasmid pUC-TetVP16' and the 'target plasmid pUC-Top10'. For abbreviations see Figure 2.

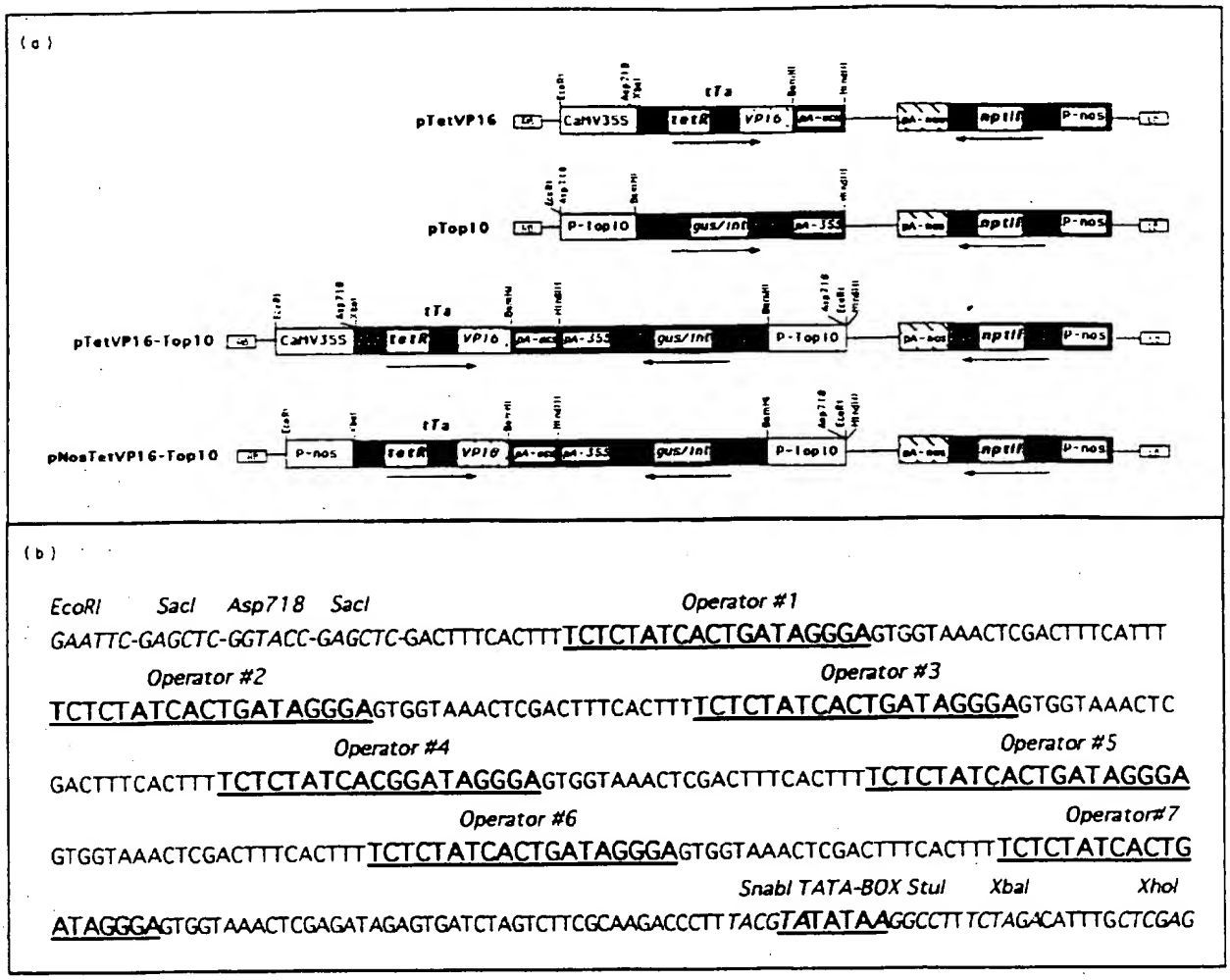


**Figure 2.** Constructs.
(a) Diagram of the chimeric genes used for the establishment of a Tc-responsive promoter in transgenic plants. pTetVp16 contains the transcriptional transactivator gene (*tTA*) under the control of the CaMV 35S promoter. pTop10 encodes the *gus/int* gene under the control of the chimeric promoter consisting of seven *tet* operator sites 5' to a TATA-box (TATATAA). pTetVP16-Top10 contains both chimeric genes on one T-DNA. pNosTetVP16-Top10 differs from pTetVP16 with regard to the promoter (P-Nos) driving *tTA* expression. RB, right border; LB, left border; pA, polyadenylation signal. P—promoter: *npt*II, neomycinphospho-transferase; *ocs*, octopine synthase; *nos*, nopaline synthase. *gus/int*: β-glucuronidase with an intron (Vancanneyt *et al.*, 1990). Arrows indicate the transcriptional orientation.
(b) Sequence of the target promoter P-Top10. The sequences of the operators and the TATA-box are underlined, recognition sequences for restriction sites are written in italics.

### *Activity of the target promoter P-Top10 during the regeneration process*

In order to characterize the function of tTA and its target promoter in stably transformed plants we cloned the respective chimeric genes on binary vectors which were designed for *Agrobacterium tumefaciens*-mediated gene transfer (Bevan, 1984). As a first step we replaced the *gus* gene of the reporter construct by a modified version that contains an intron (Vancanneyt *et al.*, 1990). Thus Gus activity of transformed plant tissue can be monitored very early after the co-cultivation step, because the gene is not properly expressed in *Agrobacterium*. Four different transgenic tobacco lines were generated. Figure 2 depicts the different chimeric genes that were cloned into BIN19. pTetVP16 encodes only the 'activator construct' whereas pTop10 carries only the 'target construct' adjacent to the chimeric kanamycin-resistance gene. pTetVP16-Top10 contains both constructs on the same T-DNA. To avoid a potential influence of the CaMV 35S enhancer on transcription from the target promoter P-Top10 the reporter construct was placed downstream of the chimeric *tTa* gene. A tail-to-tail orientation of both genes was chosen as a further precaution. pNosTetVP16-Top10 differs from pTetVP16-Top10 with regard to the promoter driving the expression of tTA: whereas pTetVP16-Top10 encodes

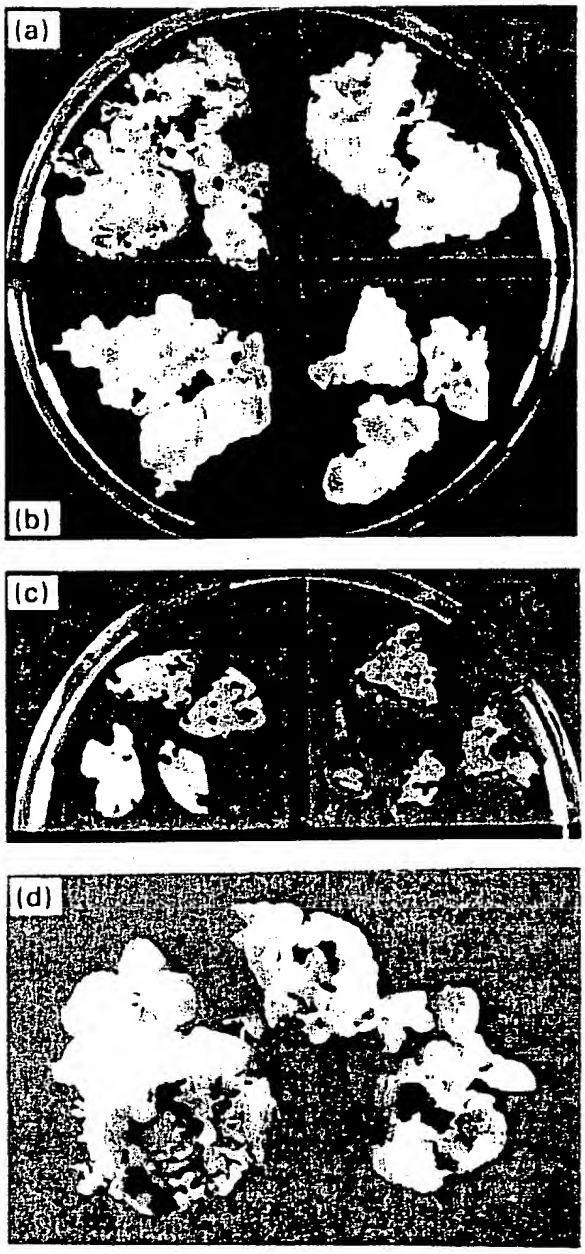


**Figure 3.** Gus expression in regenerating transformants.
(a) TetVP16-Top10, 36 days after transformation; (b) Top10, 36 days after transformation; (c) CaMV 35S/Gus–int, 20 days after transformation; (d) pNosTetVP16-Top 10, 74 days after transformation. The Tc concentration in the medium was 1 mg $l^{-1}$.

tTA under the control of the CaMV 35S promoter, the Nos promoter (An *et al.*, 1986) was used in pNosTetVP16-Top10.

As shown in Figure 3(a), Gus activity is only detectable in explants transformed with pTetVP16-Top10, when grown in the absence of Tc. No activity is seen in leaf discs incubated on Tc-containing medium. Explants from the transformation with pTop10, a plasmid that contains only the 'target construct', did not express the *gus* gene, neither in the presence nor in the absence of Tc (Figure 3b). Explants from a control transformation with the CaMV 35S promoter driving the *gus/int* gene (Vancanneyt *et al.*, 1990) proved the specificity of the Tc effect (Figure 3c). The staining of explants from the pNosTetVP16-TOP10 transformation (Figure 3d) revealed Gus activity only in the callus stadium, where the Nos promoter is active enough to direct sufficient levels of *tTA* expression. In shoots, however, no activation of the *gus* gene was observed.

### *Regulation of the target promoter P-Top10 in transgenic plants*

Gus activities of 60 shoots from TetVP16-Top10 plants, 50 shoots from Top10 plants and 20 shoots from Nos-TetVP16-Top10 plants were determined. After 30 min the reaction of TetVP16-Top10-derived extracts was stopped.

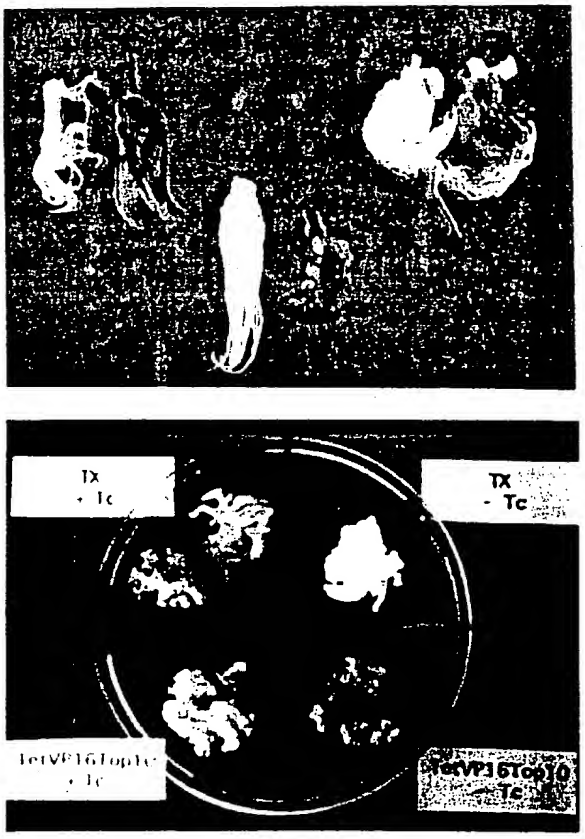


**Figure 4.** Inhibition of Gus activity in tissues grown on Tc-containing medium.
Upper panel: Gus expression in roots of plants nos 22, 24 and 30, grown either in the absence (–) or presence (+) of 1 mg $l^{-1}$ Tc. Lower panel: transformed plants were re-regenerated either in the presence or absence of Tc. Plant TX contains the Tc-inducible Triple-Op promoter in front of the *gus* gene.

**Table 1.** Gus activities of nine highest expressing TetVP16-Top10 plants and a representative Triple-Op/gus plant (TX)

| Transformant | pmol 4 MU $min^{-1}$ $mg^{-1}$ protein |
|---|---|
| 5 | 820 |
| 6 | 1550 |
| 13 | 3190 |
| 22 | 1780 |
| 24 | 1060 |
| 30 | 1470 |
| 42 | 2045 |
| 43 | 6070 |
| 47 | 7530 |
| TX | 13 000 |

Gus activity was determined using the fluorometric assay of Jefferson (Jefferson *et al.*, 1987). One leaf was taken when shoots had just formed small roots on kanamycin containing medium.

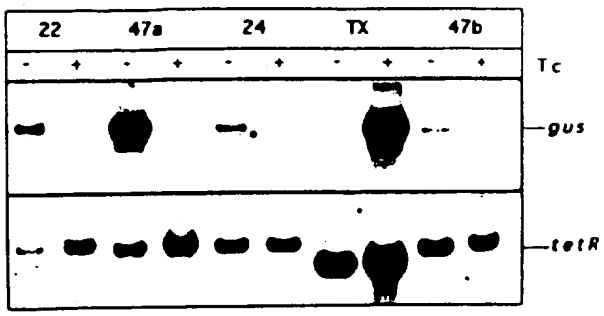


**Figure 5.** Northern blot analysis of TetVP16-Top10 plants.
RNA was prepared either directly from plants grown under axenic conditions on 2MS medium (lanes marked –) or after infiltration with 1 mg $l^{-1}$ Tc and 48 h incubation on Tc-containing medium (lanes marked +). In lanes TX+ and TX– RNA of a transgenic plant containing the *gus* gene under the control of the Tc-inducible TX-promoter was loaded to demonstrate the reciprocal effect of Tc. The blot was first probed with a restriction fragment containing the *gus* coding region, and subsequently with a 695 bp tetR fragment.

The average Gus activity was calculated to be 660 pmol 4 MU $min^{-1}$ $mg^{-1}$ protein (U). Expression levels of the nine highest expressing plants varied between 7530 and 820 U (Table 1). These plants were kept for further analysis. Extracts from Top10 transformants and NosTetVP16-Top10 transformants were incubated for 12 h in order to detect even low amounts of activity. Under these conditions only four Top10 plants showed a Gus activity of ca. 50 U. Activities in all the other shoots were indistinguishable from activities measured in untransformed control plants (10 U). To assess the effect of Tc on Gus activity of TetVP16-Top10 plants, we first compared tissues which were newly formed either in the presence or absence of Tc. Thus, we avoided potential complications due to the stability of the Gus protein. The upper panel of Figure 4 shows roots from plants nos 22, no. 24 and 30; the lower panel shows regenerating shoots from explants taken from plant no. 47 and from a transgenic plant expressing the *gus* gene under the control of the Triple-Op promoter (TX) which is negatively regulated by TetR (Gatz *et al.*, 1992). Even after staining overnight no detectable Gus signal was observed in roots grown in 2MS medium with 1 mg $l^{-1}$ Tc indicating a very stringent regulation. Quantitation using the fluorometric assay (Jefferson *et al.*, 1987) revealed that tissue formed in the presence of Tc (roots, callus, regenerating shoots) never showed higher Gus activity than untransformed controls, even when the extracts were incubated for more than 12h. Under these conditions, the Triple-Op promoter, which is inducible by Tc, gives values between 100- and 250-fold regulation. As the Top10 promoter in the presence of Tc yields no activity above the background measured in untransformed control plants, we cannot calculate an equivalent numerical value for the efficiency of regulation. When shoots that had formed on Tc were placed on 2MS without Tc, Gus activity reaccumulated, which shows that the regulation is reversible.

The explants shown in the lower pannel of Figure 4 indicate that Gus expression levels are sensitive to Tc not only in newly formed shoots, but also in leaf explants, which had expressed Gus activity before being put on Tc. We confirmed this on the RNA level by Northern blot analysis of RNA from leaves being vacuum infiltrated with and without Tc (Figure 5). As expected, plants nos 22, 47 and 24 showed a Tc-mediated decrease in mRNA accumulation. Plant TX, which contains the *gus* gene under the control of the TetR-regulated promoter, shows the positive effect of Tc in that system. At the level of total RNA no background activity could be detected, with longer exposures showing only signals originating from cross-hybridization to ribosomal RNA. Whereas no. 47 showed the expected expression levels comparable with the levels given by the CaMV 35S promoter (lane 47a, see Table 1), plants nos 22 and 24 showed unexpectedly low mRNA levels. As the RNA was isolated 3 months after quantitation of Gus expression levels, it seems that the target promoter gets silenced when the plants grow older. This was confirmed by analyzing RNA of plant no. 47 1 year after the transformation. The decrease in the amount of expression is demonstrated in lane 47b (Figure 5). This instability of the activity of the target promoter was observed in all our transformants. Rehybridization of the Northern blot with the *tetR* probe revealed, that the silencing of the promoter was not due to reduced expression of *tTA* mRNA. Gus activities of 10 kanamycin-resistant seedlings from plants nos 13, 30 and 47 were determined to be 430 U, 50 U and 250 U, respectively, which still is significantly lower when compared with the expression levels of the young transformants (Table 1). All of them showed again stringent sensitivity to Tc.

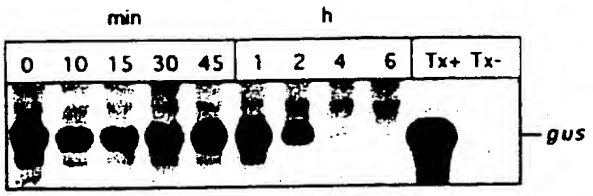


**Figure 6.** Kinetics of Tc-dependent decrease in gus mRNA abundance. Leaves form plant TetVP16-Top10#13 were infiltrated with 1 mg $l^{-1}$ Tc in 50 mM sodium citrate and incubated on MS medium. RNA was extracted after the time points indicated above the lanes. In lanes TX+ and TX– RNA from a transgenic plant containing the *gus* gene under the control of the Tc-inducible TX-promoter was loaded. The blot was probed with a restriction fragment containing the *gus* coding region.

### *Time course of Tc-dependent Gus expression*

The time course of Tc action on the steady-state levels of *gus* mRNA was analyzed in leaves of plant no. 13 which were treated with 1 mg $l^{-1}$ Tc by vacuum infiltration. RNA was extracted after 10 min, 15 min, 30 min, 45 min, 1 h, 2 h, 4 h and 6 h. After a lag time of 1 h, the RNA decayed with a half life time of less than 1 h (Figure 6).

The time course of Tc action on the steady-state levels of Gus protein was analyzed at the whole plant level. Two cuttings of plant no. 22 were transferred into hydroponic culture in the greenhouse. When the plants had reached a height of 40 cm (10 leaves), Tc was added to one cutting. At regular intervals (every third or fourth day) samples were taken from the eight upper leaves of each plant and their Gus activities were determined. Values of the untreated plant were used as the 100% reference point each day. The mean value for each series of measurements was plotted against time on a half logarithmical scale (Figure 7). After 4 weeks, Gus activity had dropped down to 1% of the initial activity, but did not decrease further. In the experiment shown here, the half life of the Gus protein in green tobacco plants is ca. 3–4 days. However, the half life depended on the size of the plant. When plants with only four leaves were treated with Tc, Gus activity had a half life of approximately 1 day (data not shown).

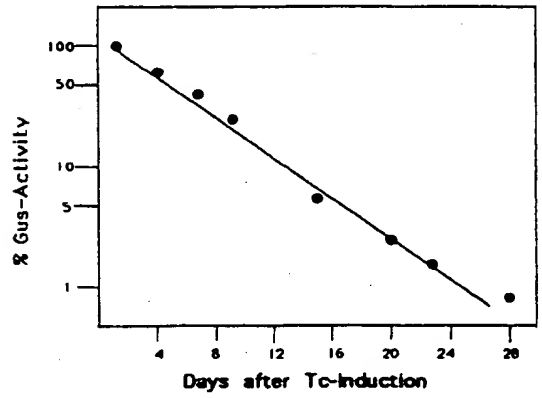


**Figure 7.** Kinetics of Tc-dependent decrease of Gus enzyme activity. Two cuttings of TetVP16-Top10#22 were grown in hydroponic culture in the greenhouse. 3, 7, 9, 15, 20, 23 and 28 days after the onset of Tc treatment of one plant; samples of eight leaves of each cutting were taken. The graph shows the mean values. Each day samples of the untreated control were calculated to be 100%.

### *Characterization of tTA expression in transgenic plants*

Transgenic TetVP16 and TetVP16-Top10 plants were analyzed for the expression of tTA. Extracts from 20 TetVP16 plants were subjected to gel shift analysis and eight plants showing strong signals were chosen for RNA analysis (Figure 8a). *Tetvp16* RNA (*tTA* RNA) is clearly detectable, though less abundant than *tetR* mRNA, which was isolated from a transgenic plant transformed with pTet1 (Gatz *et al.*, 1991). pTetVP16 and pTet1 are identical, except for the 381bp extension of the *tetR* coding region. To correct for the amount of RNA loaded we rehybridized the blot with the *S4* probe, which encodes a ribosomal protein (Devi *et al.*, 1989). Even though slightly more RNA was loaded for plant TetVP16#17, it appears to encode the highest amount of tTA and may be used in the future for subsequent transformations with chimeric genes under the control of the Top10 promoter. tTA expression

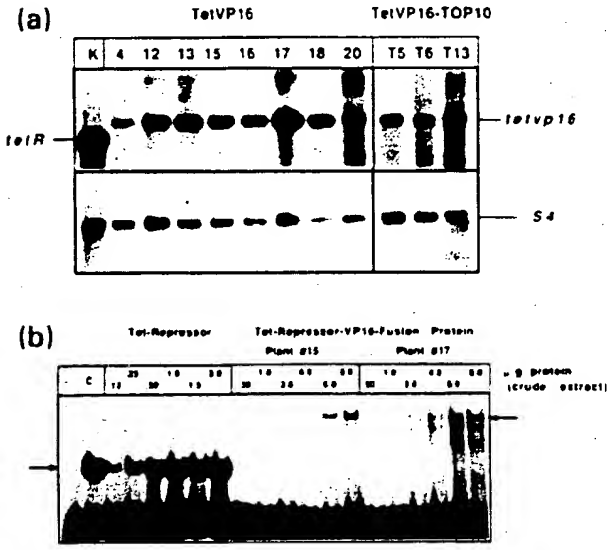


**Figure 8.** Analysis of tTa expression in plants.
(a) Northern blot analysis of TetVP16 and TetVP16-Top10 tranformants. Lane K contains RNA from the Tet1#2 transformant expressing high amounts of TetR (Gatz *et al.*, 1991). The blot was first probed with a restriction fragment containing the *gus* coding region and subsequently with the probe for the ribosomal protein S4.
(b) Gel shift analysis to compare TetR and tTA abundance in crude extracts from transgenic plants. End-labeled operator fragment (6 fmol) was incubated with increasing amounts of protein extract. Numbers above the lanes indicate the amount of protein in µg. In lane C TetR purified from *E.coli* extracts was loaded. Arrows point to the protein-DNA complexes.

levels of three plants transformed with pTetVP16-Top10, which showed different Gus expression levels (Table 1) were in the same range. The gel shift analysis provides biochemical evidence for tTA expression in transgenic plants (Figure 8b). Owing to the change in molecular weight and charge the complex has a reduced electrophoretic mobility as compared with the Tet repressor–operator complex.

## Discussion

We have established a regulated promoter for transgenic plants whose activity is turned off in the presence of low amounts of Tc. Regulation is based on an activation mechanism: a chimeric protein (tTA) consisting of the Tn*10*-encoded Tet repressor (TetR) fused to the activation domain of a eucaryotic transcriptional activator (VP16) (Gossen and Bujard, 1992) was expressed in plants. When bound to an array of *tet* operator sequences upstream of the TATA-box of the CaMV 35S promoter it activates transcription *in vivo*. In the presence of Tc, which prevents tTA from binding to the operator sequences, the promoter is inactive. In contrast, the principle of our previously established Tc inducible system (Gatz *et al.*, 1992) is based on repression. The Tn*10*-encoded Tet repressor sterically interferes with the establishment of a functional transcription initiation complex if three operator sites are suitably engineered in the vicinity of the TATA-box of the CaMV 35S promoter. Addition of Tc induces gene expression because it prevents TetR from binding. For both expression systems the features of the Tn*10*-encoded TetR—namely its capacity to bind to operator DNA and the action of Tc to interfere with binding (Hillen *et al.*, 1984)—were exploited. This discussion includes a comparison of the promoter-activating versus the promoter-repressing system for the transcriptional regulation in plants.

### *Transient assays*

Transient assays proved that tTA is functional in plants. This was expected in view of the observation that the acid domain of VP16, originally encoded by *herpes simplex* virus, can activate transcription in a variety of organisms like yeast (Berger *et al.*, 1992), mammalian cells (Sadowski *et al.*, 1988), insects (Wampler and Kadonaga, 1992) and plants (McCarty *et al.*, 1991). As in the other systems, we observe 'squelching' effects at higher tTA concentrations, which indicates that tTA interacts with some component of the transcriptional machinery, even if it is not bound to operator DNA (Berger *et al.*, 1990). At optimal concentrations, the activity of the tTA-driven promoter is higher than the activity mediated by the CaMV 35S promoter. However, its background activity in the absence of the activator was rather high (10–18% of the optimal tTA-dependent activity). It might well be that the sequence between the operators upstream of the TATA-box contains a cryptic activation site that is recognized in protoplasts. We have shown previously that the operator sequence itself does not mediate activation (Frohberg *et al.*, 1991). In addition, sequences between +1 and −50 in the same vector do not confer promoter activity (data not shown). Upon addition of Tc activity of the reporter construct dropped, similar to results obtained earlier in stably and transiently transformed Hela cells (Gossen and Bujard, 1992). However, at 2 mg $l^{-1}$ Tc, a concentration which causes maximal induction in the repressed system, tTA is not completely inactivated. These results are in contrast to comparable transient expression experiments in mammalian cells, where even lower Tc concentrations completely inhibit tTA-dependent activation (Gossen and Bujard, unpublished results). As in stably transformed plants even 1 mg $l^{-1}$ Tc is sufficient for complete inactivation of tTA (see below), we would assume, that the incomplete inactivation is not due to a reduced affinity of tTA for Tc. We would rather favor the explanation that at the high concentrations of transactivator and reporter DNA present in a transient expression system, the tTA–Tc complex might still cause some activation due to unspecific binding to the introduced target plasmid. In conclusion, these results indicate that the tTA- based system is not appropriate for the regulated expression in transient systems. In contrast, we do not find any background activity and maximal inducibility by Tc in transient assays when TetR is used to to repress transcription (Gatz *et al.*, 1992).

### *Stable transformants*

The problems encountered in transient assays were not observed in stably transformed plants. Forty-six transgenic plants containing the Top10 construct did not show any detectable Gus activity, four plants showed low activity, probably due to the integration of the T-DNA in the proximity of enhancer elements that might interact with the TATA-element of the target promoter. If the chimeric *tTA* gene was introduced along with the target promoter, 46 out of 60 kanamycin-resistant plantlets expressed the *gus* gene if grown in the absence of Tc. The difference in the expression levels between the inactive state and the active state of this system is difficult to quantify because Gus activity in the presence of Tc is close to zero. Similar results were obtained in HeLa cells, where luciferase activity was less than 2 U in cells grown in the presence of Tc and up to 257 100 U in the absence of Tc resulting in a regulation factor of at least $1 \times 105$ (Gossen and Bujard, 1992). Gus activity in untransformed plants and TetVP16-Top10 plants grown in the presence of Tc was 10 U in our

hands, maximal activity was 7000 U. We therefore conclude, that the amount of regulation is greater than 700-fold, but it is very likely that this is an underestimation. In contrast, the regulation of the repressor based system does not exceed 500-fold under optimum conditions. This confirms the notion that promoter-activating systems are more efficient for the tight regulation of individual genes in higher eucaryotes than regulatory systems based on sterical interference. This may be partly due to the fact that transcriptional activators have free access to their target sites, whereas repressors compete with endogenous transcription factors for binding. Thus, higher levels of a repressor protein are needed for the same degree of occupancy of target sites. In addition, 50% occupancy of binding sites can be sufficient for transcriptional activation but not for stringent repression. In this regard, the copper-controlable gene expression system for whole plants, which uses a yeast metalloresponsive transcription factor to stimulate transcription in the presence of copper is—in theory—very promising (Mett *et al.*, 1993). However, the regulation was reported to be only 50-fold under optimal conditions, partly due to residual background activity. The steroid-inducible regulatory system, which is based on the glucocorticoid transcription factor, has been shown to be functional in transiently transformed protoplasts, but not in stably transformed tissue (Schena *et al.*, 1991). If even very low amounts of a gene product might be lethal for the plant, we would suggest use of the tTA-based system rather than the inducible promoter. Gene expression can be obtained if plants are removed from Tc-containing medium. However, gene activation depends on the dilution and/or inactivation of Tc *in planta*, a process that spans at least a week and may vary with growth and light conditions. Thus, a potential disadvantage of the system is, that gene expression cannot be obtained at a precise time point. For some applications, like the regulated excision of selective markers between *lox* sites (Dale and Ow, 1991), the exact length of the induction period would not matter: explants could be regenerated on a selective medium in the presence of Tc; upon cultivating without Tc after selection, the Top10 promoter driving the *lox* recombinase would be turned on in the whole plant.

When discussing efficiencies of regulatory expression systems the expression levels of the induced stage should be evaluated. Gus activities are usually indicated in pmol 4 MU $min^{-1}$ $mg^{-1}$ protein (Unit). However, data on the widely used CaMV 35 promoter activity, for instance, vary over a broad range. CaMV 35S promoter mediated Gus activities were reported to be 113 000 Units (average of 10 plants, Benfey *et al.*, 1989), 321 U (one selected plant, Jefferson *et al.*, 1987), 9000 U (average of 15 plants, Sanger *et al.*, 1990), 500 U (highest expressing plants, Comai *et al.*, 1990) and 130 000 U (one selected plant, Keil *et al.*, 1989). In our hands, the CaMV 35S promoter and its Triple-Op derivative yield activities between 10 000 and 30 000 U in representative plants. The activities mediated by the tTA-dependent Top10 promoter ranged between 1200 and 7000 U. Given the above-mentioned variations of the units determination we cannot relate that to the 1200 U reported for the copper-inducible system. Direct comparison of the tTA-based system with the CaMV 35S promoter, however, allows us to state that the activity is three to five times lower than the activity of the CaMV 35S promoter and its Tc inducible-derivative P-Triple-Op. This feature might be due to the closely linked location of the reporter construct and the activator construct. Because of the counterselection against high tTA expression (see Figure 8), only plants with T-DNA integrations in less highly expressed locations might have survived the regeneration process. It remains to be investigated if a second transfection of the TetVP16#17 plant with a Top10 construct yields higher activities. However, the initial activity was not stable. One year after the transformation event, plants kept in tissue culture consistently did not synthesize more than 400 U Gus. As shown by Northern blot analysis (Figure 6) this was not due to reduced transcription of the *tTA* gene. Also, in the progeny we could not detect the initial amounts of Gus activity. It remains to be investigated, whether methylation within this region or co-suppression due to multiple T-DNA insertions are responsible for the inheritable reduced stability.

### *Kinetics of the regulation*

The possibility of switching off transcription from one specific promoter should allow analysis of mRNA or protein decay rates of individual genes. Thus the tTA-dependent promoter might provide a good alternative to the use of general inhibitors like actinomycin D and cycloheximide. When Tc was applied through vacuum infiltration into single leaves, gene expression of the inducible promoter reached maximal levels within less than 30 min, indicating a rapid inactivation of TetR (Gatz *et al.*, 1991). Tc-infiltrated leaves of a TetVP16-Top10 plant showed a response after a lag time of 1 h. From there on *gus* mRNA disappeared with a decay rate of less than 1 h. As decay rates depend on the age of the material, we would however suggest that, if the stabilities of two mRNAs are to be compared, both should be put under the control of the Top10 promoter and integrated simultaneously into one transgenic plant.

Application of Tc through the roots of a TetVP16-Top10 plant led to a decay of the protein following first order kinetics already 1 day after Tc treatment. The half life of the protein was 3–4 days in a plant 40 cm high. When younger plants were used, the protein disappeared three times faster. Again, we would suggest, that the system is

very useful for the direct comparison of the stabilities of two proteins.

### *Expression of tTA in transgenic plants*

The transient assays already indicated, that high levels of *tTA* expression might lead to the inhibition of transcription due to squelching effects. Consistently we found that transgenic plants synthesize less mRNA for tTA than for TetR (Figure 8a). The gel shift analysis (Figure 8b) allows a rough estimation of the difference between TetR and tTA expression levels: 0.12 µg of the extract derived from plant Tet1#2 encoding TetR retard about the same amount of operator encoding DNA as 6 µg of the TetVp16#17 extract, indicating that tobacco plants tolerate about 50-fold less tTA than TetR. However, this estimation is based on the assumption that tTA binds operator DNA with a similar affinity as TetR, and that it is as stable in the crude extract as TetR. The Nos promoter, which is reported to be 30-fold less active than the CaMV 35S promoter (Sanders *et al.*, 1987), is too weak to synthesize sufficient amounts of tTA in leaves. The blue staining we observed in callus of NosTetVP16-Top10 plants proves that the construct is functional. We have found no direct comparison of Nos promoter activity in leaves and callus in the literature. However, it has been described, that the Nos promoter is under developmental control (An *et al.*, 1988) as well as wound inducible and auxin inducible (An *et al.*, 1990). Therefore it is very likely, that its expression is higher in callus than in leaves. This indicates that tTA levels have to exceed a certain threshold to be able to drive expression from the target promoter.

In conclusion, we have established a Tc-dependent expression system in transgenic plants and demonstrated its potential to regulate efficiently expression of a transgene. For some applications, this system, which is negatively regulated by Tc, might be superior to the previously established Tc-inducible promoter (Gatz *et al.*, 1992): (i) it is particularly useful if the stringency of the regulation has to be very high; (ii) It can be used for the study of mRNA and protein decay rates.

## Experimental procedures

### *Plants, bacterial strains and media*

*Nicotiana tabacum* W38 was obtained through 'Vereinigte Saatzuchten' (Ebstorf, Germany). Plants in tissue culture were grown under a 16 h light/8 h dark regime on Murashige and Skoog medium (Murashige and Skoog, 1962) containing 2% sucrose (2 MS). *Escherichia coli* strain DH5α (Bethesda Research Laboratories, Gaithersburg) was cultivated using standard techniques (Sambrook *et al.*, 1989). *Agrobacterium tumefaciens* strain C58CX1 containing pGV2260 (Deblaere *et al.*, 1985) was cultivated in YEB medium (Vervliet *et al.*, 1975).

### *Reagents*

DNA restriction and modification enzymes were obtained from Boehringer Mannheim (Ingelheim, Germany) and New England Biolabs (Danvers, USA). Chemicals were obtained through Sigma Chemical Co. (St. Louis, USA) or Merck (Darmstadt, Germany).

### *Recombinant DNA techniques*

Standard procedures were used for recombinant DNA work (Sambrook *et al.*, 1989).

### *Constructs*

A diagram of the final constructs is shown in Figure 2. pTetVP16: pTet1 (Gatz *et al.*, 1991), which contains the *tetR* coding region between the CaMV 35S promoter and the octopine synthase (*ocs*) transcriptional terminator, was digested with *Bam*HI and *Sph*I and treated with T4-Polymerase to generate blunt ends. Religation of the vector resulted in the loss of the *Xba*I, *Sal*I, *Pst*I and *Sph*I sites between the coding region and the *ocs* transcriptional terminator. The resulting plasmid pTet1Δ was digested with *Xba*I and *Bam*HI in order to excise *tetR* sequences downstream from amino acid 3. The coding sequence of tTA was gained by *Xba*I/*Bam*HI digestion of pUHD 15-1 (Gossen and Bujard, 1992) and ligated into pTet1Δ cut with *Xba*I and *Bam*HI to yield pTetVP16. pUC-TetVP16: The *tTA* gene under the control of the CaMV 35S promoter and the *ocs* transcriptional terminator was excised from pTetVP16 as an *Eco*RI/*Hin*dIII fragment and inserted into pUC18 cut with *Eco*RI and *Hin*dIII (Figure 1). pUC-Top10: pUC-Top10 was derived from pIGF107 (Gatz *et al.*, 1991), which contains the coding region of the bacterial *chloramphenicol acetyl transferase* (*cat*) gene under the control of a modified CaMV 35S promoter. This promoter contains a number of additional unique restriction sites between positions −1 and −53. The plasmid was linearized with *Spe*I at position −53, the protruding ends were filled in using Klenow DNA polymerase, and a synthetic 55 bp oligonucleotide (Gatz *et al.*, 1991) encoding two operators and a *Bgl*II site was inserted. The promoter fragment was cloned as an *Eco*RI/*Xho*I (fill-in) fragment into pGus (Köster-Töpfer *et al.*, 1989) cut with *Eco*RI and *Sma*I. The resulting plasmid pSpe-Gus was cut with *Asp*718 and *Bgl*II(fill-in) and ligated with the *Asp*718/*Xho*I (fill-in) fragment from pUHC 13-4 (Gossen and Bujard, 1992), so that the CaMV 35S enhancer was replaced by seven *tet* operators. In the course of this step part of the 55 bp oligonucleotide was unintentionally deleted, so that seven *tet* operator sequences (instead of nine) were left. The sequence of this promoter (P-Top10) is shown in Figure 2(b). pUC-Top10-Gus/int: the coding region of the β-glucuronidase (*gus*) gene and the nopaline synthase (*nos*) polyadenylation signal from pUC-Top10 was excised with *Bam*HI and *Hin*dIII and replaced by the coding region of a modified *gus* gene containing an intron (Vancanneyt *et al.*, 1990) and the CaMV 35S polyadenylation signal. pTop10: The *gus/int* gene under the control of the synthetic Top10 promoter was cloned as an *Eco*R/*Hin*dIII fragment into pBIN19 (Bevan, 1984) cut with *Eco*RI and *Hin*dIII. pTetVP16-Top10: a *Hin*dIII linker was cloned into the *Eco*RI (fill-in) site of pUC-Top10-Gus/int. The *gus/int* gene under the control of the synthetic Top10 promoter was cloned as a *Hin*dIII fragment into pTetVP16 *Hin*dIII. For further experiments we chose a plasmid that contained the *tTA* gene and the *gus* gene tail to tail (Figure 2). pNosTetVP16: pTetVP16 was cut with *Eco*RI and *Asp*718 (fill-in).

The vector fragment was ligated with an *EcoRI/PstI* (made blunt ended with T4-polymerase) fragment encoding the Nos promoter (An *et al.*, 1986). The *gus(int)* gene under the control of the synthetic Top10 promoter was cloned as a *HindIII* fragment into pTetVP16 *HindIII* yielding pNosTetVP16-Top10 (Figure 1).

### *Binding studies with plant extracts*

An operator containing restriction fragment was excised with *EcoRV* and *BglII* from pLUP-1 (Frohberg *et al.*, 1991), end-labeled by filling in the protruding ends in the presence of [$\alpha$-$^{32}$P]dATP using Klenow-Polymerase and separated from the vector by polyacrylamide gel electrophoresis and subsequent elution. Binding reaction and gel electrophoresis were done as described (Gatz *et al.*, 1991).

### *Transient expression in tobacco protoplasts*

Isolation and transformation of protoplasts was essentially as described (Gatz *et al.*, 1991)

### *Northern blot analysis*

Total RNA from leaves was prepared according to Logemann *et al.* (1987). Blotting and hybridization was carried out as described (Heyer and Gatz, 1992).

### *Assays for Gus activity*

For the fluorometric Gus assay, explants were homogenized and incubated with the substrate 4-methylumbelliferyl-β-D-glucuronide at 37°C. Quantification of the fluorescence was done according to Jefferson *et al.* (1987). Protein concentrations were determined according to Bradford (1979).

For *in vivo* staining, intact plant material was vacuum infiltrated with 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexylammonium) and incubated overnight at 37°C.

### *Tobacco transformation*

Transformation of tobacco plants was carried out using the *Agrobacterium tumefaciens* leaf disc technique as described by Rosahl *et al.* (1987).

### *Application of Tc to the plants*

Plants were regenerated on 1 mg $l^{-1}$ Tc. Vacuum infiltration of single leaves was done as described (Gatz *et al.*, 1991). For Tc uptake through roots, plants were cultivated in a beaker containing Hoagland buffer and 1 mg $l^{-1}$ Tc, which was changed every other day. Oxygene was supplied through an aquarium pump.

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